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A light and transmission electron microscopical characterization of contact sensitivity in the mouse.

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A LIGHT AND TRANSMISSION ELECTRON MICROSCOPICAL
CHARACTERIZATION OF CONTACT SENSITIVITY
IN THE MOUSE

by

SHEILA MARY FAIRLEY MCKEE-PROTOPAPAS

A Thesis
submitted to the Faculty of Graduate Studies
through the Department of Biology
in Partial Fulfillment of the
Requirement for the Degree of
Master of Science at
the University of Windsor

Windsor, Ontario, Canada

1983

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ABSTRACT

In this study the male F₁ progeny of a cross between hr/hr and BALB/c mice were used to study contact sensitivity to 2,4-dinitro-1-fluorobenzene (DNFB). Correlative light and transmission electron microscopy were used, as well as adenosine triphosphatase (ATPase) and immune region associated antigen (Ia) staining techniques, both specific for epidermal Langerhans cells. In preliminary work the hairless mouse exhibited a negligible reaction whereas all haired strains studied displayed a positive response.

It was found that an infiltration composed mostly of mononuclear cells, presumably T cells, and a few polymorphic leukocytes began in the dermis at about 6 h post-challenge. The intensity of this infiltrate increased until 48 h after challenge when it occupied almost the whole dermis. The heaviest dermal infiltrate was always associated with hair follicles.

At 12 h post-challenge mononuclear cells were observed in the epidermis. Their numbers increased until at 48 h an acanthotic epidermis filled with invading cells existed. The cells had migrated from the dermis into the epidermis and at 48 h and 72 h they could be seen sloughing off with the stratum corneum.

Ultrastructural changes in the epidermis began at 6

h after challenge with the gradual disappearance of tonofilaments from keratinocytes. Desmosomal connections appeared reduced and keratohyalin diminished by 24 h. These changes may be associated with increased cellular proliferation. Mononuclear cells were observed in the epidermis and in cellular debris being sloughed off at 24 h and 48 h. By 72 h after challenge the epidermis was thickened and beginning to repair itself.

The ATPase stain showed a normal distribution of Langerhans cells at 12 h post-challenge, a slight concentration at 24 h and clumping at 48 h, particularly around hair follicles. The Ia marker stain indicated that not all of these clumped ATPase positive cells were Langerhans cells, since there was only a slight increase in Ia positive cells in the vicinity of the hair follicle at 48 h post-challenge; they were not macrophages or B cells either, as they are Ia positive. Electron microscopic examination of ATPase stained sheets revealed that cells with all the characteristics of Langerhans cells, except granules, and small cells resembling mononuclear cells exhibited a positive reaction. Eosinophils observed in the same epidermis lacked the lead precipitate. ATPase positive cells clumped around the hair follicle are probably mononuclear cells, presumably T cells.

These studies have provided an insight into the microscopic nature of the contact sensitivity reaction which as been shown to occur in the ear and the back in mice. In addition, they have supplied evidence to support the suggestion that the hair follicle is somehow involved in the reaction. Finally an intriguing phenomenon has been revealed in the comparison of ATPase and Ia stained epidermal sheets.

DEDICATION

This thesis is dedicated to my husband Les.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Dr. J.E.J. Habowsky for his untiring help and guidance over the past two years. A most sincere thank you is extended to Ken Baker who has rendered assistance countless times. Together Dr. Habowsky and Ken have made learning here an exciting and enjoyable experience. I would like to thank Dr. D. des S. Thomas and Dr. N.F. Taylor for reviewing and evaluating this thesis. I would also like to express my appreciation to Mrs. Johanna Belanger for her excellent work in typing this manuscript. A final thank you to my husband and my parents for their unwavering confidence and love.

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LIST OF ABBREVIATIONS

Ag	- antigen
APC	- antigen presenting cell
ATPase	- adenosine triphosphatase
B cell	- bone marrow derived lymphocyte
CS	- contact sensitivity
DNCB	- 2,4-dinitro-1-chlorobenzene
DNFB	- 2,4-dinitro-1-fluorobenzene
DTH	- delayed-type hypersensitivity
EDTA	- ethylenediaminetetraacetic acid
FITC	- fluorescein isothiocyanate
Ia	- immune region associated
LC	- Langerhans cell
LM	- light microscopy
PBS	- phosphate buffered saline
RT	- room temperature
T cell	- thymus derived lymphocyte
TEM	- transmission electron microscopy
UA	- uranyl acetate

INTRODUCTION

In this study correlative light and electron microscopic studies will be used to elicit more information about the complex contact sensitivity (CS) reaction. Although several hypotheses have been advanced as to its nature, the exact mechanism of contact sensitivity remains obscure (47). If determined, it may be that relief could be found for the problematic contact sensitivities encountered in industry (reactions to industrial chemicals), in the cosmetics field, in topical drug therapy and also in matters as mundane but troublesome as poison ivy (70).

Contact Sensitivity

Hypersensitivity is an immune response induced by an antigen (Ag) or a hapten (a partial Ag) that can lead to damage to an organism (70). It is also commonly known as allergy (16). There are two categories of hypersensitivity: immediate-type and delayed-type (DTH). Immediate reactions are brought about by antibodies, peak at 1-3 h, and usually only last 10-15 h (16). DTH is a cell-mediated immune response and it is not apparent until 1-2 days after contact with the Ag. It can last several days or weeks (16). Immediate-type

hypersensitivity can be transferred to naive recipients with serum, whereas DTH can only be transferred with living leukocytes (13,16,44).

Several reactions can be ascribed to DTH (70): allergies to chemicals and drugs, homograft rejection, the tuberculin reaction, resistance to infection and viruses and contact sensitivity (15).

Contact sensitivity (CS) is also referred to as allergic contact dermatitis. It is characterized at the gross level by edema and erythema, and at the light microscopic level by an infiltration of mononuclear cells (19). CS is brought about by contact of the Ag or hapten (sensitizer) with intact skin.

There is a wide range of chemicals that can be used to elicit CS. They are generally of low molecular weight and examples include picryl chloride, oxazolone, mercuric chloride, 2,4-dinitro-1-fluorobenzene (DNFB) and 2,4-dinitro-1-chlorobenzene (DNCB) (70). In this study DNFB is the sensitizing agent used. It combines with components of the epidermis to form a hapten-protein conjugate which is the actual Ag (70).

In order to produce a CS reaction the sensitizer is first introduced to the skin (induction or sensitization) and then reapplied at a lower concentration several days later (elicitation or challenge). The sensitizing dose

must be low enough that it will not cause an immediate irritant reaction which may mask the DTH reaction under investigation.

Applying the chemicals to the surface of intact skin (painting) is the most common method of sensitization and is also used in this study (16,36,70). Application can be repeated, if desired, to obtain a heightened response, although a single painting will suffice (2).

Approximately 4-5 days after the last sensitizing dose the elicitation dose is applied to the surface of the skin (16,36,70). A positive reaction is visible at the elicitation site 24-48 h later.

It is felt that on introduction to the skin during sensitization the hapten combines with epidermal proteins to form an Ag complex. The next step requires a cell to "present" Ag to the lymphocytes responsible for CS. This immunocompetent cell binds the Ag in the skin, travels to the draining lymph node via afferent lymphatics, and presents the Ag to lymphocytes (47). An alternative is that some Ag arrives unbound in the lymph node and is presented to the lymphocytes by resident dendritic cells (47). Both methods appear to be utilized. The lymphocytes are thus primed to respond specifically to that Ag.

Upon challenge an antigen presenting cell (APC) again picks up Ag and migrates to the lymph node. The Ag could also travel there unbound, or primed lymphocytes circulating in the blood or lymphatics may recognize the Ag at the challenge site. The lymphocytes respond by proliferation and aggregation at the challenge site and the hypersensitivity reaction results.

In most DTH reactions the macrophage is the APC. Two steps are involved in the presentation process: recognition of Ag and attachment to the macrophage membrane (38).

Langerhans Cell

In CS reactions the Langerhans cell (LC) appears to be the APC (3,61,67). Epidermal LCs are located in the stratum spinosum of humans and guinea pigs and in the stratum basale of mice (5). They have also been observed crossing the basement membrane (24), an activity which suggests that they may be migratory.

First discovered by Paul Langerhans in 1868 (31), the LC has been difficult to visualize in LM preparations. Birbeck and Breathnach (8) detected a unique granule, visible only at the electron microscopic level which has since served to identify the cell ultrastructurally. The granule is trilaminate with

central striations and is generally rod or racquet shaped (47,56). Other ultrastructural features of the LC are numerous dendrites, a lobulated nucleus, and a lack of the tonofilaments and desmosomes characteristic of keratinocytes (75). LCs also do not contain the melanin or melanosomes which are characteristic of melanocytes. Cells possessing all the LC features except granules are called indeterminate cells; most investigators feel they are an immature form of the LC.

Langerhans cells possess several characteristics which enable the investigator to quantitate LCs and study their distribution using light microscopy. Except for Langerhans' use of gold chloride on post-mortem tissue (31), LC "markers" were unknown. Wolft and Winkelmann (73,74) showed that a stain utilizing the adenosine triphosphatase (ATPase) present on the LC membrane was specific for LCs and indeterminate cells in the epidermis. They were able to quantitate LCs and the method has since been modified to stain epidermal sheets (4,27,35). The stained tissue can also be embedded for transmission electron microscopic observation which shows the technique to be specific for LCs; keratinocytes have a very light lead precipitate at their periphery but LCs show a dark, black concentration of lead associated with their plasma membrane. Melanocytes do not exhibit ATPase activity.

Immune region associated antigen (Ia) is a surface glycoprotein present on groups of immunologically active cells (49). An indirect immune staining procedure and fluorescein isothiocyanate (FITC) or peroxidase are used to visualize the cells. Macrophages, bone marrow-derived lymphocytes (B cells, which produce antibody), a small number of thymus-derived lymphocytes (T cells) (49) and dendritic cells from the spleen, liver, lymph node and thymus (63) also possess Ia on their plasma membranes. It was shown to be present on LCs by several research teams concurrently (30,50,51,64). Nordlund and Ackles (43) then modified the technique for epidermal sheets.

Another antigen marker, OKT6, is found on the surface of LCs (12) and Stingl, et al., (65) have demonstrated the presence of receptors for the Fc portion of IgG and a component of complement (C3). Two cytoplasmic stains can be used to study LCs by LM: S-100 (41,42) and β -glucuronidase (34). All of these techniques have proved useful in the quantitation of LCs and the study of their distribution.

The fact that LCs do induce CS has been confirmed by several workers, either by attempting to produce a CS reaction in skin naturally or artificially deficient in LCs (67,68,69) or by monitoring epidermal proliferation (10) which may be a manifestation of CS

(28). In addition, Shelley and Juhlin (55) studied ten known contact sensitizers and found that LCs took up all ten. They suggested the role of a reticulo-epithelial system for the LC network, similar to the reticulo-endothelial system (54). Further evidence results from the work of Silberberg-Sinakin, et al. (62) who sensitized guinea pigs with ferritin and consequently found ferritin-containing LCs in draining lymph nodes.

In a series of experiments Silberberg and colleagues (57,58,59,60) found LCs apposed to mononuclear cells after challenging guinea pigs sensitized to numerous chemicals. This apposition was not seen in controls or in irritant reactions to higher doses of the same chemicals. It is most likely that this close contact between LCs and mononuclear cells occurs at the time of Ag presentation.

When lymphocytes have been stimulated by contact with LCs they produce inflammatory lymphokines which include interleukin 2, vascular permeability-increasing factor, migration inhibition factor (45) and chemotactic factor (72). These cause the invasion of more mononuclear cells, macrophages and some polymorphonuclear leukocytes (polymorphs).

Detection and Measurement of Contact Sensitivity

Gross morphologic observations have proved useful in determining the extent of a CS reaction in humans and guinea pigs (19). The degree of edema and erythema are the parameters used, an increase in either indicating a stronger response.

Several techniques are available to detect a positive CS reaction in the mouse. Most investigators use the method of Asherson and Ptak (1) which consists of eliciting the response in the ear and then measuring the thickness of the ear with an engineer's micrometer. If the ear has swollen significantly a positive reaction is assumed. Similar to this method is the measurement of swelling in the tail and footpad (18) after subcutaneous challenge.

Various problems arise in the employment of these procedures. Because the ear of the mouse is thin and fragile, accurate measurement of its thickness is difficult. In an investigation of this technique Rowden (48) reported that it was impossible to obtain consistent results if the ear was measured by different technicians.

The uncertainty of accuracy when estimating the degree of CS by measurement of tail and footpad swelling occurs not only due to the difficulty of actual

measurement but also because the skin in these areas is atypical. In tail skin the distribution of LCs is irregular so it is possible that the reaction taking place is abnormal. Because footpad skin is much thicker than body wall skin the reaction as it occurs there is probably unrepresentative of skin as a whole.

These three methods rely on the edema resulting from trauma to the tissue as being an accurate indication of the degree of CS obtained. CS can also be assessed by light microscopy (2,19,46). The pattern for the CS reaction is the same in all species studied, notably human, guinea pig, and mouse. The difference occurs in the time course the reaction takes; in mice the maximum can be reached by 24 h post-challenge (46), whereas humans may not exhibit a peak response until 96 h (70).

Many attempts have been made to characterize the cells of the mononuclear cell infiltration that occurs during a CS response. It was determined that B cells are not necessary (17,37,66), but a specific subset of T cells (T_{dth}) is responsible for the reaction (16,25,32,71).

Preliminary Studies

Initially it was felt that the mouse was incapable of mounting a DTH response to a contact sensitizing

agent. Munoz (40) states that any response observed at 24 to 48 h post-challenge is a remnant of an immediate type reaction. The erythema and induration characteristic of the CS reaction in humans and guinea pigs are not obvious in mice, thus leading to false negative evaluations. It has since been determined that CS does occur in the mouse but the early experiments have probably led to the fact that most studies of the CS response are performed on humans and guinea pigs.

In this laboratory the hairless mouse (hr/hr) has been used extensively in studies of the skin. Because of the similarity of its integument to that of human it was felt that a study of CS in the hairless mouse could be enlightening. However, in preliminary work the hairless mouse displayed what could be considered a negligible reaction. A few mononuclear cells appeared in the dermis and in only one isolated instance were mononuclear cells found in the epidermis. Haired strains, C3H, BALB/c, and F₁ (hr/hr x BALB/c), all exhibited a positive reaction.

A characterization at successive time points using transmission electron microscopy (TEM) has not been attempted using the mouse as an experimental animal. In this study 3,6,12,24,48 and 72 h post-challenge have been chosen as representative of a time course. Preliminary

work using C3H mice has shown that the response begins at 3-6 h and peaks at 24-48 h with 72 h representing a reparative stage.

There are conflicting reports as to the effect of anatomical site of challenge on the response in the mouse. Eisen (16) states that the reaction occurs only in the ear whereas Crowle (13) uses flank skin to study CS. In comparing the back and the ear in preliminary studies using F₁ (hr/hr x BALB/c) mice a strong CS reaction in the back was found which was identical to that of the ear when examined by light microscopy (LM). In the following study the back was used for most investigations; however, when staining epidermal sheets for LCs the ear was employed.

With the preliminary results on hand a full investigation of CS in the skin of the mouse using light and transmission electron microscopy has been conducted.

Mouse Epidermis

Skin is divided into a lower layer, the dermis, and an upper layer, the epidermis (39). In the dermis fibroblasts secrete collagen to form collagen fibres. These and the elastic fibres abundant in dermis give skin its characteristic strength and flexibility. Mast cells and macrophages or histiocytes are also present in the

dermis. Capillary plexes and both sensory and autonomic nerves supply the skin at the level of the dermis.

At the interface between the dermis and the epidermis is the basement membrane (39). Electron microscopic (EM) studies show that the basement membrane consists of the basal lamina, lamina lucida, and basal cell plasma membrane (11). The basal lamina appears as an electron dense zone with fibrous components extending a short distance into the dermis. Associated with the basal cell plasma membrane are numerous hemidesmosomes; their anchoring fibrils appear to extend across the lamina lucida, an electron lucent zone between the epidermis and the basal lamina.

Scaletta and McCallum (53) have shown that the epidermis can easily be separated from the dermis after incubation in a 20 mM solution of ethylenediaminetetraacetic acid (EDTA). Separation occurs through the lamina lucida, the basal lamina remaining attached to the dermis. The hemidesmosomes are the only epidermal cell components which undergo any ultrastructural damage. The utilization of this technique provides a valuable tool in the examination of effects of various experimental conditions on the epidermis.

The epidermis consists of four layers: the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (39). At the LM level the stratum corneum is seen distinctly as the upper layer composed of enucleate, stratified squamous cells, while the other layers are often grouped together as the Malpighian layer. It is in this Malpighian layer that cell division and migration take place, providing a continual supply of cells that are eventually sloughed off from the stratum corneum.

Transmission electron microscopy reveals the stratum basale as consisting primarily of keratinocytes (39). They possess many tonofilaments and maintain contact with each other via desmosomes (see Fig. 29a). As the cells reach the stratum spinosum small droplets of electron dense keratohyalin begin to form; these aggregate and in the stratum granulosum are seen as large granules dispersed throughout the cells. Also present in the keratinocytes of the stratum granulosum are membrane coating granules which contribute to the tough, impervious cell membrane of the corneocyte. When the cells reach the stratum corneum they have lost their nuclei and are flattened and filled with keratin (39).

Keratinocytes form the majority of cells found in the epidermis, but melanocytes, Merkel cells and LCs are also present (9). Melanocytes are located in the stratum

basale and are responsible for pigment formation. Merkel cells are infrequently detected but are associated with nerve endings and are thought to serve a nervous function (75). LC number varies in different parts of the body: hamster cheek pouch and mouse tail skin have reduced LC populations and there are no LCs in the cornea of any species (6,7).

MATERIALS AND METHODS

F₁ Mice

Male hairless mice (hr/hr) from Jackson Laboratories, Barr Harbor, Maine, and female BALB/c mice from Dominion Laboratories, Dublin, Virginia were crossed producing haired F₁ progeny. Only the males, aged 15-25 weeks were used for these studies; they were kept in plastic cages and given water and pelleted food ad libitum.

Sensitization and Challenge

For sensitization the hair of the abdomen of each mouse was removed using electric clippers. DNFB (0.5 ml of a 0.5% solution in 4:1 acetone/olive oil) was applied to the shaved area with a gloved finger.

Animals were challenged four days later at one of two sites, the posterior dorsum or the dorsal surface of the ear. The posterior dorsal skin is suitable for TEM and LM studies; however, the dorsal ear skin is better suited for processing as an epidermal sheet for ATPase and Ia staining as it is relatively hairless. This allows for better adhesion to the tape backing and the necessity of chemical depilatories, which may affect the results, is avoided. A 0.2% DNFB solution (0.5 ml) in the same vehicle was applied to the posterior dorsal skin

after hair was removed or to the dorsal surface of both ears. Animals receiving the challenge dose on the ears had been given two 0.5 ml sensitizing doses 14 h apart.

Two sets of controls were used. In one control sensitization and challenge were done using the vehicle only. In the second control challenge dose only was applied.

Animals were sacrificed by cervical dislocation at 3, 6, 12, 24, 48 and 72 h after challenge except the controls which were sacrificed 48 h after challenge. Preliminary studies showed that these times are representative of the CS reaction in the mouse, the peak occurring between 24 and 48 h.

Tissue Processing of Posterior Dorsal Skin

The challenged area of the posterior dorsum was removed and specimens 4 mm x 4 mm and 2 mm x 1 mm were used for LM and TEM, respectively.

Light Microscopy - Tissue was fixed in Bouin's fixative overnight, dehydrated in a graded series of alcohols and embedded in paraffin (Appendix I). Sections 6 μ thick were mounted on slides and stained using hematoxylin and eosin (Appendix I).

Some 1 μ sections were obtained from blocks embedded for transmission electron microscopy, stained with

toluidine blue 0 and examined by light microscopy (Appendix I).

Slides were examined using an Olympus light microscope and photographed with a Nikon 35 mm camera and Kodak plus x film (Appendix IV).

Electron Microscopy - Tissue was fixed for 2 h at 40C in Dalton's potassium dichromate buffered 1% osmium tetroxide at pH 7.3 and then dehydrated and embedded (Appendix II).

Blocks were trimmed using a Reichert TM60 trimmer and thin sectioned using a diamond knife in a Reichert OMU2 Ultramicrotome. Sections were transferred to copper grids and stained with uranyl acetate and lead citrate (Appendix II). Grids were examined in either an RCA EMU-3H or a Phillips 201 transmission electron microscope and photographed using a Phillips 35 mm camera and Kodak FGRP film (Appendix IV).

Tissue Processing of Ear Skin

During the course of the reaction the ear was observed at the macroscopic level and any changes in gross appearance were noted.

In preliminary studies it was found that the CS response elicited changes in LC distribution after 12 h so the reaction in the ear was examined at 12, 24, and 48

h after challenge.

Ears were removed and placed, dorsal side down, on the adhesive side of white "Time" tape (4). They were then cut into 2 mm x 2 mm pieces and pressed, dorsal side down, to new Time tape. Thus attached to a support backing the tissue was incubated in 20 mM EDTA at 37°C for 2.5 h (Appendix III) at which time the dermis was peeled from the dorsal epidermis which remained adherent to the tape backing.

The epidermal sheets were stained for ATPase activity or the presence of surface Ia markers (Appendix II). The ATPase sheets were examined by light microscopy. Ia sheets were examined using a Leitz Wetzler ultraviolet microscope with an epifluorescence attachment and photographed using a Nikon camera and Ilford HP5 film (Appendix IV).

To determine the specificity of the stain some sheets were incubated only in the secondary antiserum (anti-IgG).

RESULTS

The results of this study will be given under headings of the number of hours post-challenge and reported as macroscopic and light and transmission electron microscopic observations and also findings concerning the ATPase and Ia stained epidermal sheets where applicable..

Three Hours Post-Challenge

The only detectable change in the gross appearance of the dorsal side of the ear is a slight prominence of blood vessels.

Observed by light microscopy the dermis lacks mononuclear cells and the epidermis is two to three cell layers thick (Fig. 1). Deep in the dermis, at the level of the subcutaneous adipose tissue, some polymorphs are noted within the blood vessels (Fig. 2).

When examined by TEM fibroblasts and collagen are observed in the dermis. The basal lamina and hemidesmosomes are intact and the layers of the skin (stratum basale, stratum spinosum, stratum granulosum and stratum corneum) are easily distinguishable (Fig. 3). Keratinocytes maintain desmosmal contact with each other and possess numerous tonofilaments and ribosomes. In the stratum granulosum keratohyalin and membrane coating

Figure 1 LM. Three hours post-challenge: Both dermis (D) and epidermis (E) exhibit no abnormal characteristics (hf - hair follicle). 265x

Figure 2 LM. Three hours post-challenge: Deep dermal blood-vessel containing polymorphs (P). 1200x



0

Figure 3 TEM. Three hours post-challenge: Epidermis shows no signs of trauma (D - dermis, bl - basal lamina, sb - stratum basale, ss - stratum spinosum, sg - stratum granulosum, sc - stratum corneum). 11,200x



granules are abundant. LCs are found in the lower epidermis (Figs. 4a,b) and possess dendrites, a convoluted nucleus and granules but lack tonofilaments and desmosomes. The presence of a keratinocyte process underlying the LC (Fig. 4) is often noted in mouse skin.

Six Hours Post-Challenge

In addition to prominent blood vessels, there is a slight erythema of the base of the ear observed at the gross level.

In the dermis of skin prepared for light microscopic examination there are a few mononuclear cells (Figs. 5a, b); a darkly staining nucleus and little or no cytoplasm are characteristic of the morphology. In some instances these cells appear clumped together deep in the dermis, superficial to the subcutaneous adipose tissue. Some polymorphs are seen in deep dermal blood vessels.

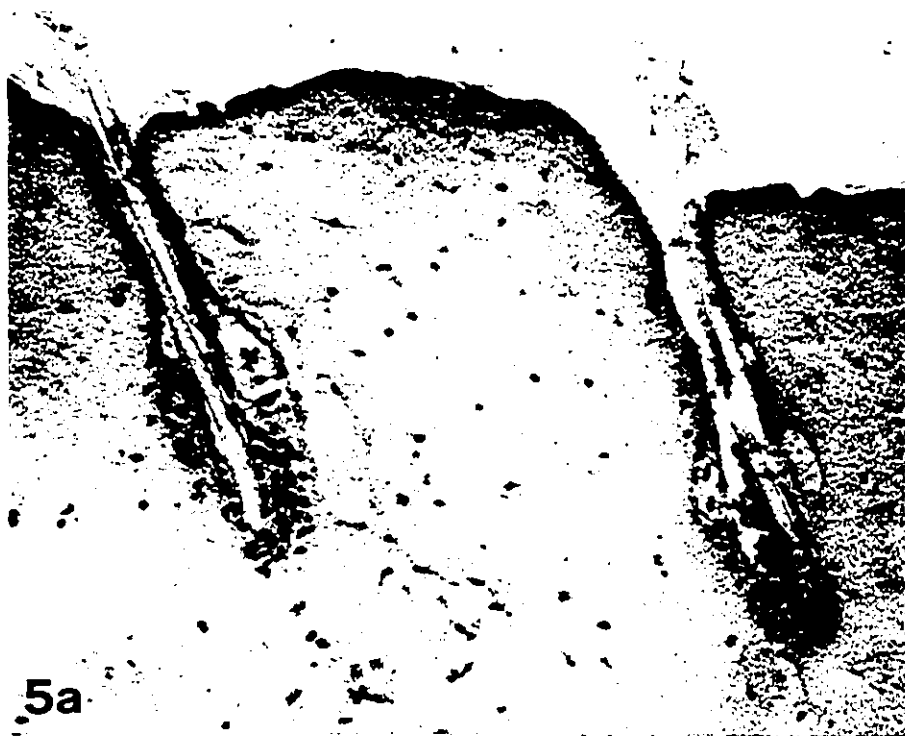
In the epidermis no signs of injury are seen; there is also no thickening or spongiosis apparent.

A large number of fibroblasts is noted in the dermis in material prepared for TEM (Fig. 6); cytoplasmic processes, many vacuoles and association with collagen fibrils are identifying characteristics. In addition, a cell was observed usually not present

Figure 4 TEM. Three hours post-challenge: (a) Langerhans cell (L) in basal epidermis (D - dermis, p - keratinocyte process). 19,900x (b) enlargement of Langerhans cell granule (gr) and centriole (c). 43,800x



Figure 5 LM. Six hours post-challenge: (a) mononuclear cells are rare in the dermis and none are found in the epidermis. 275x (b) enlargement of probable mononuclear cell (M). 1200x



5b

in the dermis (Fig. 7). It displays a polymorphic nucleus, granules of varying sizes with dense cores, as well as vacuoles containing a cloudy lipid-like substance.

The basal lamina and hemidesmosomes are intact; however, in the epidermis the keratinocytes have begun to show signs of trauma (Fig. 8). There are very few tonofilaments associated with the desmosomal connections between cells. It is also difficult to distinguish cell organelles due to the foaminess of the cytoplasm but no spongiosis is observed. The cells of the stratum basale and stratum spinosum are difficult to discern and the stratum granulosum possesses very little keratohyalin.

In one part of the epidermis a clump of unusual cells is protruding above the rest of the epidermis (Fig. 9). The cells have dark homogeneous nuclei and very little cytoplasm. They resemble keratinocytes which may be in the process of being sloughed off in an abnormal manner.

Twelve Hours Post-Challenge

The gross morphology of the ear after 12 h does not vary noticeably from that at 6 h after challenge. The blood vessels are still prominent and there is slight erythema at the base of the ear.

Figure 6 TEM. Six hours post-challenge: Fibroblasts (F)
in dermis (Co - collagen). 8200 x

Figure 7 TEM. Six hours post-challenge: Polymorph (P)
in dermis. 9500x



Figure 8 TEM. Six hours post-challenge: There is a reduction in organelle integrity in the basal cells (E - epidermis, D - dermis). 10,700x

Figure 9 TEM. Six hours post-challenge: cells (arrows) protruding above epidermis. 12,600x



Pronounced mononuclear cell infiltration of the dermis is observed by light microscopy at 12 h after challenge (Figs. 10a,b) and the invasion is especially intensive in the vicinity of hair follicles. It does not appear to be associated with dermal blood vessels. Some polymorphs are seen in the infiltrate but the majority of the cells resemble lymphocytes. Adjacent to the hair follicles some mononuclear cells may be seen in the epidermis. In places the epidermis is somewhat thickened and may bulge to some degree where the dermal infiltrate is heaviest. The stratum corneum folds down into the lower layers of the epidermis at regular intervals, however, no spongiosis is observed.

As at 6 h after challenge fibroblasts are noted in the dermis by transmission electron microscopy (Fig. 11).

Vesicles are present in some parts of the epidermis (Fig. 12). They are filled with a diffuse filamentous substance. The keratinocytes of the stratum basale and the stratum spinosum appear somewhat foamy and possess some mitochondria and fewer tonofilaments. The keratohyalin in the stratum granulosum is reduced. Extensions downward of the stratum corneum into the stratum granulosum are noted.

Figure 10 LM. Twelve hours post-challenge: (a) Dermal infiltrate (M) is associated with hair follicles (arrow indicates mononuclear cell in epidermis). 225x (b) enlargement of (a). 960x

Figure 11 TEM. Twelve hours post-challenge: Fibroblasts (F) and collagen in dermis. 5600x



A small cell with sparse cytoplasm surrounding a dark nucleus is seen in the basal layer of the epidermis (Fig. 13). Because this cell does not resemble any type of cell usually found in skin and it does look like mononuclear cells seen in other parts of the body it will be referred to as a mononuclear cell.

In the sheets of epidermis obtained from the ear and stained for ATPase intensity there is a regular distribution of dendritic ATPase positive cells (see Figs. 14a,b, which are representative of this distribution). The lead precipitate shows a stellate body with long, slender dendrites. The ATPase positive cells are dispersed uniformly throughout the interfollicular epidermis and sparsely in the hair follicles.

Twenty-four Hours Post-Challenge

Erythema of the dorsal side of the ear has spread towards the tip at this time, although it is still not pronounced. The blood vessels remain dilated.

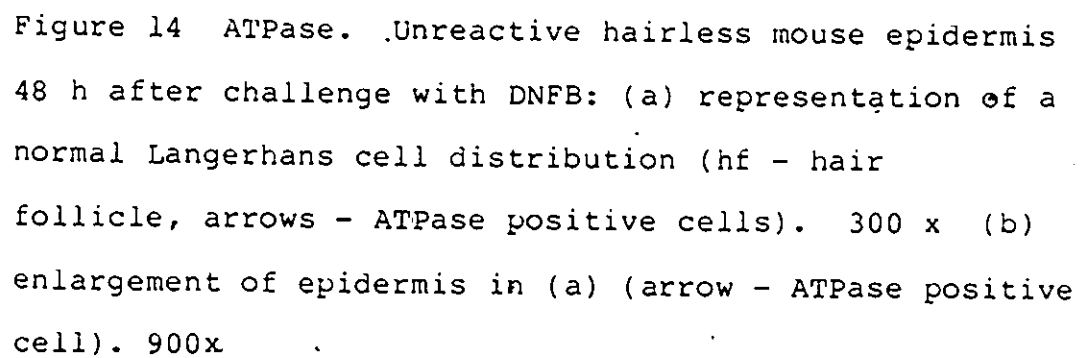
There is a pronounced dermal infiltrate seen in material prepared for LM and it is most concentrated in the region of hair follicles (Fig. 15). Most of the cells resemble lymphocytes and occasional polymorphs are seen. At the level of subcutaneous adipose tissue lymphocyte-like cells are observed in the blood vessels or lymphatics. A moderate invasion of the epidermis has taken place and it appears to have spread up from the hair

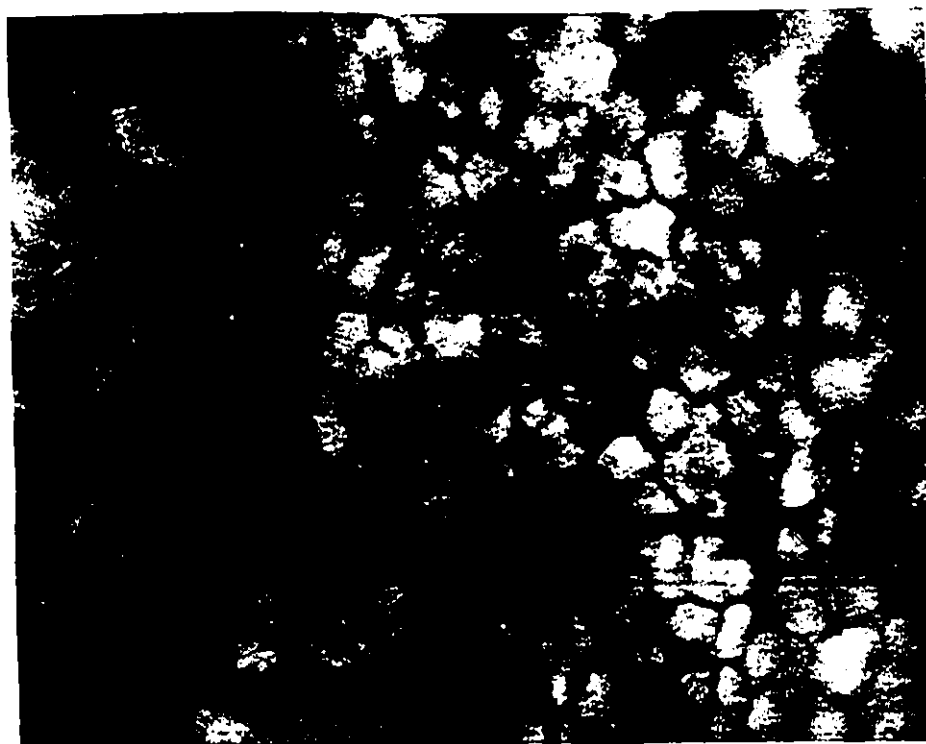
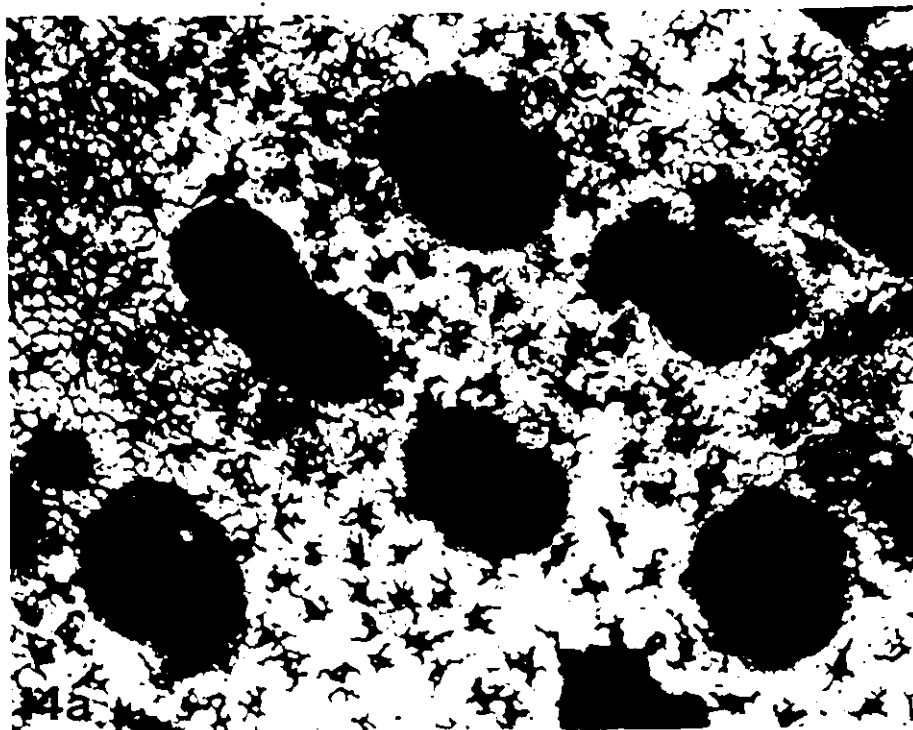
Figure 12 . TEM. Twelve hours post-challenge: Vesicles (V) are apparent in the epidermis. 8000x

Figure 13 TEM. Twelve hours post-challenge: Mononuclear cell (M) in basal epidermis(E - epidermis, bl - basal lamina, D - dermis). 17,300x



Figure 14 ATPase. Unreactive hairless mouse epidermis 48 h after challenge with DNFB: (a) representation of a normal Langerhans cell distribution (hf - hair follicle, arrows - ATPase positive cells). 300 x (b) enlargement of epidermis in (a) (arrow - ATPase positive cell). 900x





follicles. In places the infiltration forms a type of acanthosis; ie., it comprises a layer above the stratum granulosum as it is sloughed off. An intensive invasion can also cause the epidermis to bulge over the dermis (Fig. 16). Between areas of infiltration the epidermis has thickened to 5-6 cell layers but no spongiosis is observed. The stratum corneum appears to extend into the Malpighian layer in places.

Tissue examined by TEM exhibits mononuclear cells with large dark nuclei and sparse, clear cytoplasm in the dermis. The basal lamina is disrupted in places and hemidesmosomes are occasionally absent (Fig. 17). In the stratum basale tonofilaments and desmosomes are absent, whereas a few desmosomes can be seen between the keratinocytes of the stratum spinosum. In other areas occasional mononuclear cells are observed in the epidermis.

Between the stratum granulosum and the stratum corneum is a layer of unusual composition (Fig. 18). It contains a dark homogeneous substance interspersed with what appear to be nuclei and other debris and an eosinophil is observed. This layer is not continuous over the entire epidermis but appears to exist between only parts of the stratum corneum (Fig. 19). The stratum corneum exists as layers of enucleate cells and seems to extend in pockets down into the lower layers of the epidermis in areas where the heterogeneous layer

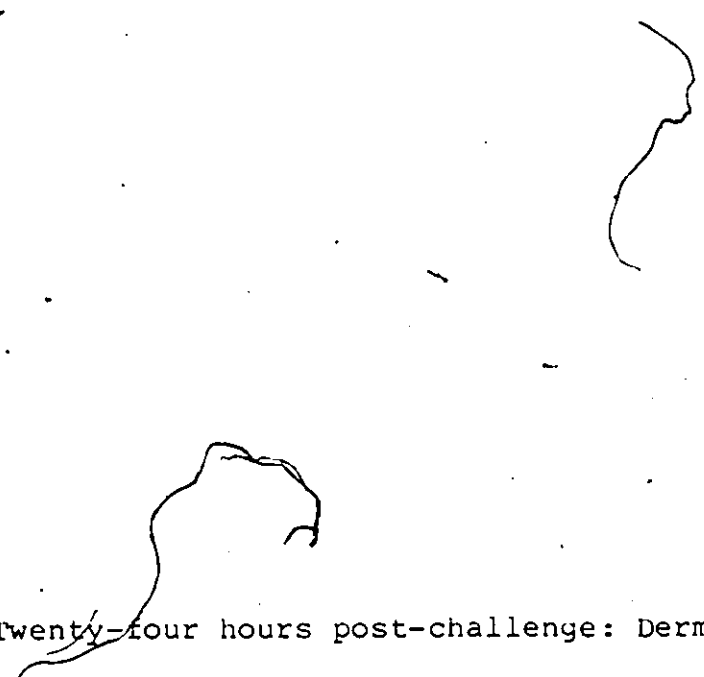


Figure 15 LM. Twenty-four hours post-challenge: Dermal and epidermal infiltrate (M) is associated with hair follicles. 215x

Figure 16 LM. Twenty-four hours post-challenge: Epidermis bulges over aggregate of infiltrating cells. 865x



Figure 17 TEM. Twenty-four hours post-challenge: Most cellular detail is lost in basal epidermal cells (tonofilaments, desmosomes and nuclei are absent) (arrow - discontinuity of basal lamina). 4500x



Figure 18 TEM. Twenty-four hours post-challenge:
Heterogeneous zone (H) between layers of stratum corneum
(Eo - eosinophil). 4800x

Figure 19 TEM. Twenty-four hours post-challenge:
Heterogeneous zone (H) between layers of stratum
corneum. 3800x



is absent. No spongiosis is observed in the epidermis.

Interfollicular ATPase positive cells at 24 h after challenge are stellate with dendrites and are dispersed evenly throughout the epidermis. However, there is a noticeable concentration of ATPase positive cells around some hair follicles. Some of these clustered cells possess dendrites while others do not, having only round ATPase positive bodies.

Forty-eight Hours Post-Challenge

The prominent blood vessels and slight erythema of the dorsal side of the ear are still apparent at the gross level.

The dermal and epidermal mononuclear cell infiltration is very heavy, the epidermis appearing to be replaced by the infiltrate in many areas (Fig. 20). The cells of the infiltrate resemble lymphocytes and a few polymorphs are present. The infiltration extends down into the hair follicles and acanthosis is wide-spread. In toluidine blue O sections a dark-staining homogeneous substance is located between collagen fibres in the dermis.

In the dermis as observed by transmission electron microscopy, there are numerous fibroblasts and abundant

collagen (Fig. 21). Between the cells and the collagen there is a dark homogeneous substance. Mononuclear cells are also apparent in the dermis.

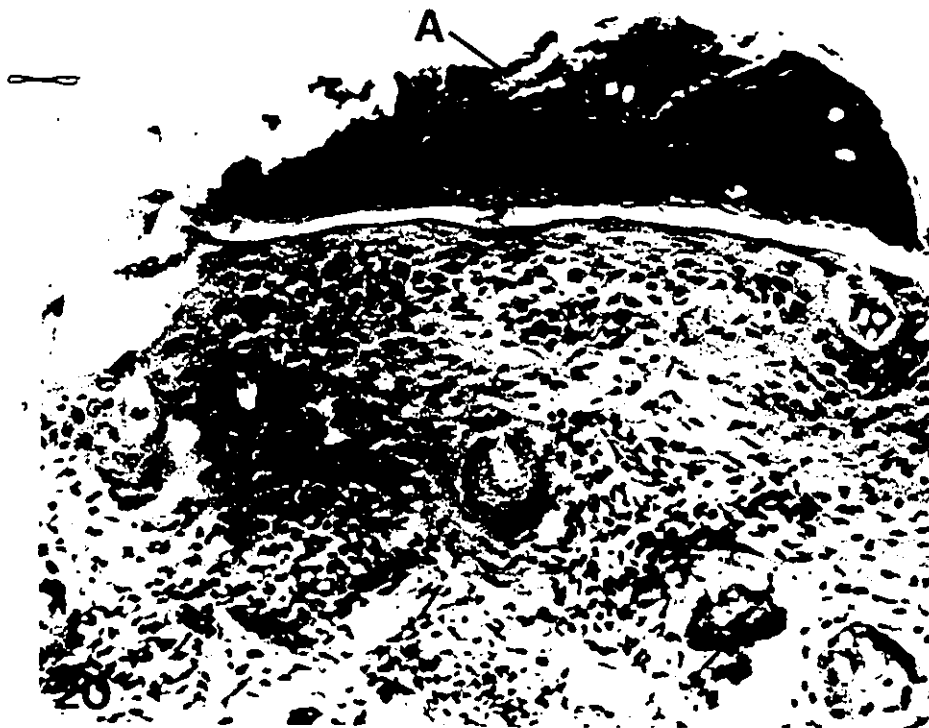
It is difficult to determine where the dermis and epidermis meet (Fig. 22a). The basal lamina appears to have been partially obscured and in places disrupted, although hemidesmosomes are visible. The basal keratinocytes are foamy and have no tonofilaments. In the stratum granulosum keratohyalin is sparse and appears less electron dense than usual. The stratum corneum is very thick and tortuous and contains a great deal of debris, similar to the unusual layer described at 24 h (Fig. 22b). The thickness of the epidermis varies from 1-2 cells to much thicker than normal.

ATPase positive cells are somewhat rounded and their dendrites seem to be shorter; some cells are lacking dendrites altogether. Around the hair follicles ATPase positive cells are clearly clumped (see Fig. 23, which is representative of clumping). There are some dendritic cells but many are without dendrites and most have round cell bodies.

As noted using ATPase stain the Ia positive cells are distributed evenly throughout the interfollicular epidermis (Fig. 24a); these cells are dendritic and have stellate to round bodies (Fig. 24b). However, rather than clumping around the hair follicles the Ia positive

Figure 20 LM. Forty-eight hours post-challenge:
Acanthotic epidermis (A) overlying dermis which has
marked infiltration (M). 230x

Figure 21 TEM. Forty-eight hours post-challenge:
Numerous fibroblasts (F) in dermis with abundant
collagen. 4500x



7

Figure 22 TEM. Forty-eight hours post-challenge: (a)
The boundary between dermis (D) and epidermis (E) is
indistinguishable and cells of the epidermis have lost
most of their desmosomes and tonofilaments.
9800x (b) Debris (d) is often found in the stratum
corneum. 6600x

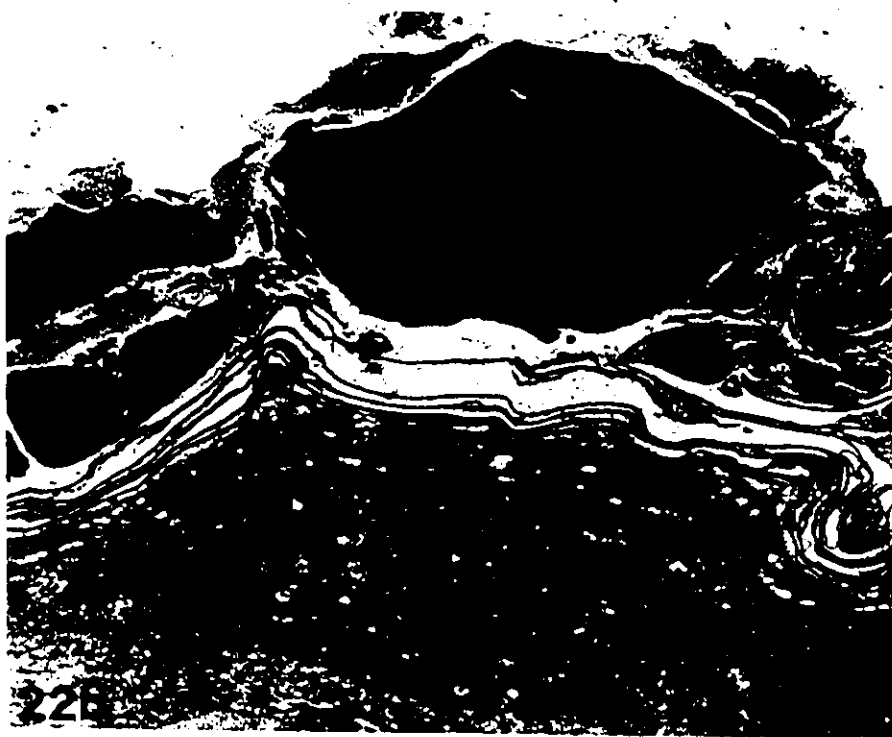
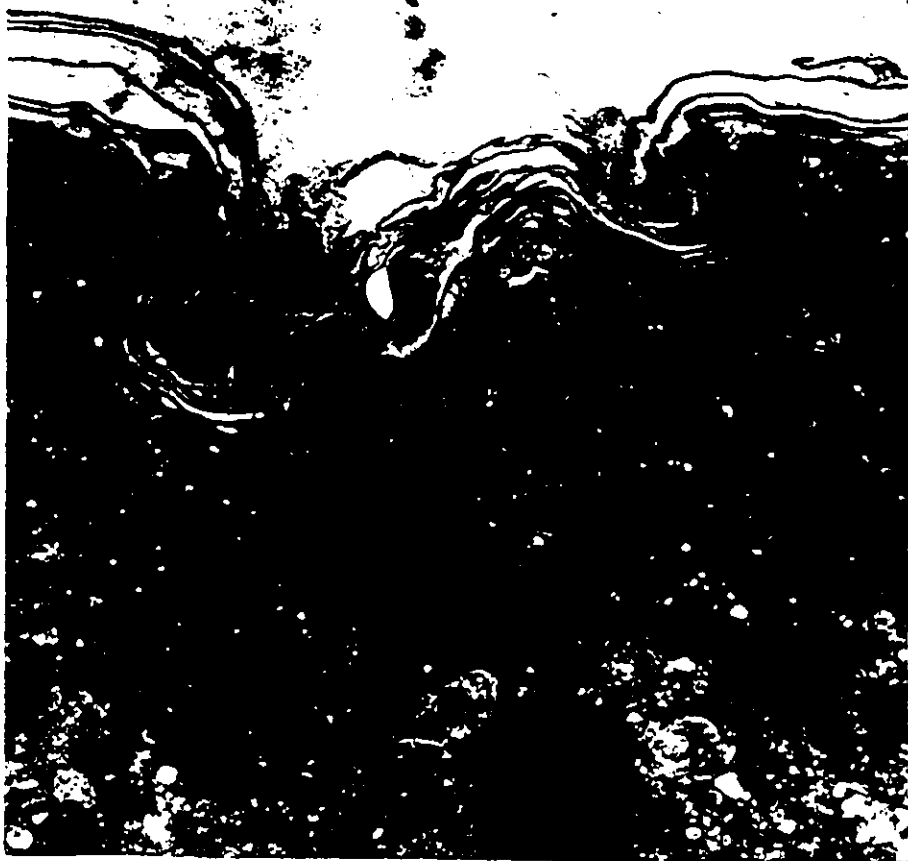


Figure 23 ATPase. Clumping of ATPase positive cells (arrows) near a hair follicle 72 h after challenge with DNFB to a C3H mouse (representative of distribution at 48 h in BALB/c mouse). 215x

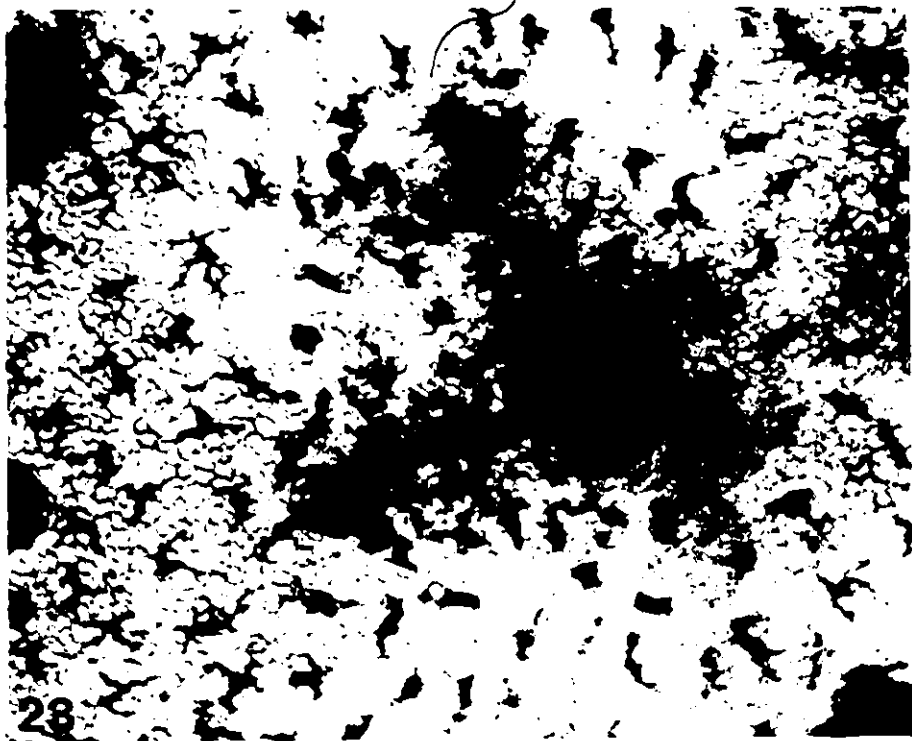




Figure 24 Ia antigen. Forty-eight hours post-challenge:
(a) Fluorescent dendritic Langerhans cells (arrows) and
a hair follicle (hf). 225x (b) enlargement of (a) 530x



cells are only slightly more concentrated in these regions (Fig. 24a). All of the Ia positive cells at the hair follicle are dendritic.

Seventy-two Hours Post-Challenge

In tissue studied by LM the dermis contains a few mononuclear cells, usually in the vicinity of the hair follicles or associated with regions of pronounced epidermal infiltrate (Fig. 25). The invasion by mononuclear cells in the epidermis appears moderate and no longer actually replaces the epidermis. The infiltrate is sloughed off dorsal to the epidermis which is thickened, but not spongy.

When examined by TEM the dermis resembles that found at 48 h after challenge. Collagen and fibroblasts are abundant and a dark homogeneous substance is located in the spaces between the fibres and cells.

The basal lamina is indistinguishable as is the division between dermis and epidermis. The keratinocytes are foamy and ill-defined and are lacking in tonofilaments (Fig. 26). The stratum corneum is seen extending under the rest of the epidermis in places and it lifts off sections of the acanthotic epidermis (Fig. 27). There is a layer of sloughed off cellular debris located above the stratum corneum and detached from the





Figure 25 LM. Seventy-two hours post-challenge: Very little infiltrate is present in dermis and epidermis is thickened (A - sloughed off acanthotic epidermis). 270x





L

Figure 26 TEM. Seventy-two hours post-challenge:
Thickened epidermis exhibits spongiosis (S). 6500x



Figure 27 TEM. Seventy-two hours post-challenge: Part of the epidermis being lifted off by infoldings of stratum corneum (SC). 5300x

27

SC



skin in most areas.

Control-Vehicle Only

Mononuclear cell infiltration does not occur in the dermis or epidermis 48 h after challenge with the vehicle only and the epidermis is 2-3 cell layers thick (Fig. 28).

In control skin studied by TEM the dermis contains collagen and fibroblasts and the matrix is clear and free from any debris (Figs. 29a,b). The basal lamina and hemidesmosomes are intact and the layers of the skin are easily distinguishable. The cells of the stratum basale possess abundant tonofilaments and desmosomes. The stratum spinosum, stratum granulosum and stratum corneum show no signs of injury or trauma.

Control-Challenge Only

When observed by LM scattered mononuclear cells are apparent in the dermis. The epidermis is of normal thickness although it seems to be detached from the dermis in places (Fig. 30). Spongiosis in the basal epidermis can be seen after staining with toluidine blue O.


Transmission electron microscopic examination of skin treated only with the challenge dose reveals a

Figure 28 LM. Control - vehicle only, 48 h post challenge: Dermis and epidermis exhibit no indications of trauma or injury. 260x



28

Figure 29 TEM.. Control - vehicle only, 48 h post challenge: Both epidermis (a) and dermis (b) resemble those of normal skin (Co - collagen, D - dermis, sb - stratum basale, ss - stratum spinosum, sg - stratum granulosum, sc - stratum corneum d - desmosome, t - tonofilaments). 11,000x



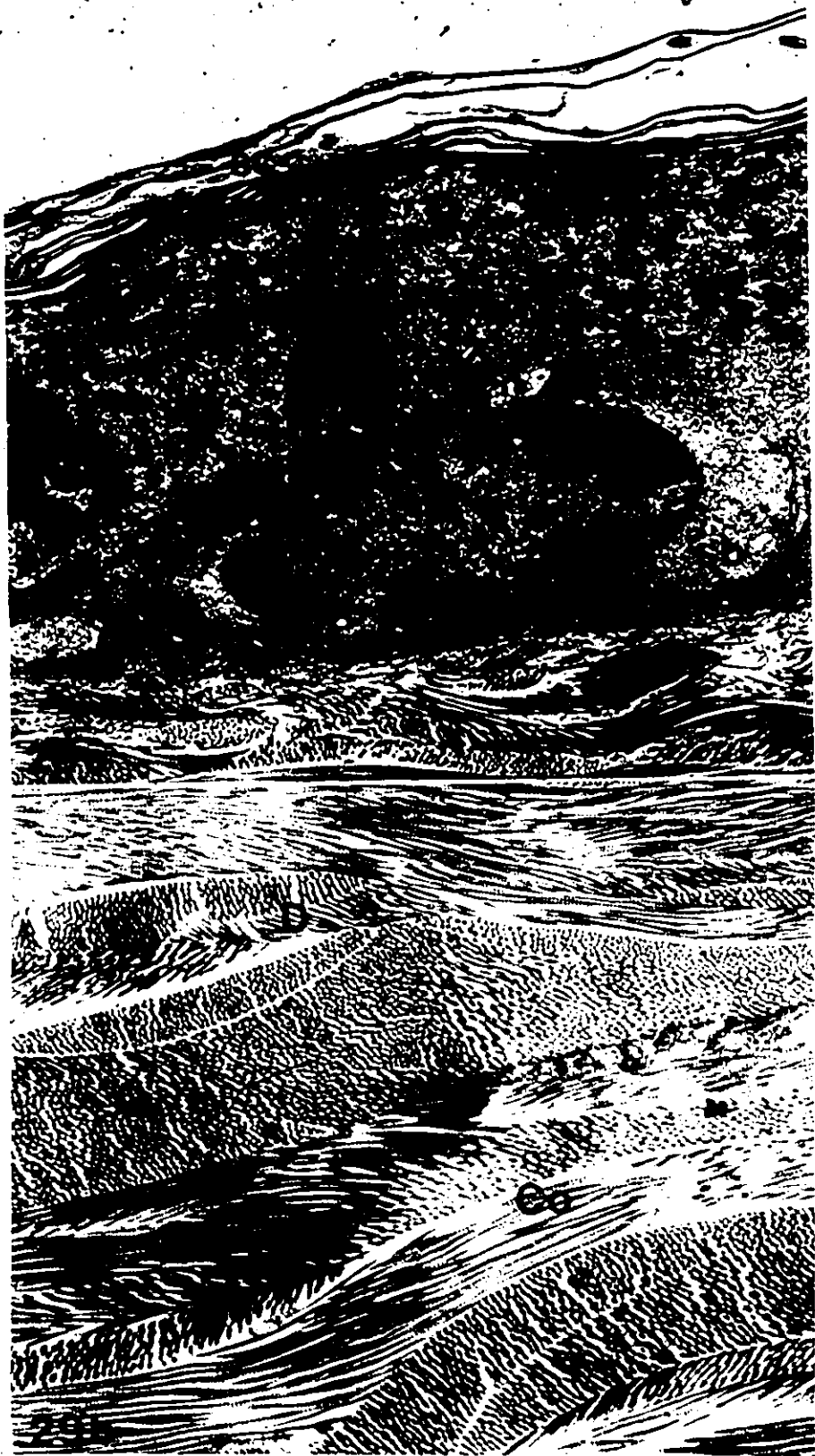


Figure 30 LM. Control - challenge only, 48 h post challenge: Parts of epidermis are detached from dermis by the fusion of vesicles (arrow). 245x



dermis that exhibits no abnormal characteristics (Fig. 31). Located between the dermis and the epidermis are some vesicles. In some instances the epidermis appears to have been lifted off the dermis due to these vesicles. The lower layers of the epidermis are spongy in some areas (Fig. 31) and the spaces between the cells are bridged by cytoplasmic processes which terminate in desmosomal connections. Apart from this abnormality the epidermis displays no unusual characteristics.

Ia-Secondary Antiserum Only

No fluorescent dendritic cells are seen in mouse ear epidermal sheets after incubation with only the secondary antiserum (anti-IgG). The hair shaft fluoresces demonstrating that the FITC present in the secondary antiserum does function.

A very low number of small round fluorescent cells is observed in the interfollicular epidermis.

Figure 31 TEM. Control - challenge only, 48 h post challenge: Spongiosis (S) apparent in basal layer of an otherwise normal epidermis. 4300x



31

DISCUSSION

The results of this study have shown that the CS reaction in the mouse generally resembles that in human and guinea pig (70). It is apparent that the mononuclear cells first appear in the dermis associated with hair follicles and then migrate into the superficial dermis, through the basal lamina and into the epidermis. There they most likely secrete lymphokines and other chemicals which cause the keratinocytes to undergo profound changes and possibly migrate towards the surface at a faster rate, taking the offending chemical with them. Together with the damaged keratinocytes the mononuclear cells and polymorphs are sloughed off the top of the epidermis and the epidermis begins to repair itself.

There is a great deal of evidence to suggest that the invading mononuclear cells are T cells, specifically Tdth (16). Thus, the mononuclear cells referred to in this study are probably T cells although they cannot be unequivocally identified by the techniques employed here.

It can be stated that this series of studies has provided an insight into the microscopic changes associated with the CS reaction in the mouse. Because only human and guinea pig had been studied in such detail, it was apparent that such a study was necessary.

In addition, these investigations have supplied evidence to support the suggestion that the hair follicle is somehow involved in the CS response. The mononuclear cell infiltrate seen by LM (dermal) and correlative ATPase experiments (epidermal) is always centred around the hair follicle(Figs. 15,23). The sensitizing chemical probably penetrates the epidermis via hair follicles and remains most concentrated there. Macher and Chase (33) stated that the sensitizer remaining in the epidermis is most potent in eliciting the CS reaction.

Gross observations of the response to challenge of the dorsal side of the ear with DNFB revealed only that the blood vessels begin to dilate as early as 3 h after challenge and remain dilated until 48h. The erythema that occurred was slight. No scaling or other indications of injury were visible. Because of the apparent inconstancy of the measurement of ear swelling (48), which has previously been used as an indication of the degree of reactivity, it was not attempted.

In contrast with mice, humans and guinea pigs show marked, gross changes during the development of a CS response. There is conspicuous erythema, induration and swelling of the skin site involved, parameters

often used to judge the degree of CS obtained (43).

The fact that mice do not show these pronounced changes has, in the past, led to the belief that the mouse is incapable of mounting a DTH-response to a contact sensitizing agent (40).

Three Hours Post-Challenge

Both light and transmission electron microscopic examinations reveal that the dermis and epidermis show all the characteristics of normal mouse skin (Figs. 1,3). Mononuclear cells not are seen in either the dermis or the epidermis.

In an irritant reaction (ie., an immediate reaction which results from the use of a high dose of sensitizing agent) polymorphs are the cells responsible for the resultant dilation of blood vessels and edematous swelling. The presence of a few such cells at 3 h after challenge could mark the onset of early swelling and possibly the enhancement of the infiltration of lymphocytes. In addition, some polymorphs are invariably seen at the site of CS reactions (22).

Six Hours Post-Challenge

Some scattered mononuclear cells are seen in the dermis at 6 h after challenge (Fig. 5) and the polymorphs

remain in deep blood vessels associated with the subcutaneous adipose tissue. The fact that no spongiosis is observed contrasts with the general view that basal spongiosis is one of the first morphologic changes to take place in epidermis after challenge with a contact sensitizer (19).

Fibroblasts appear to be abundant in the dermis. One of the gross morphologic changes associated with CS is induration. This is most likely due to an increase in collagen fiber synthesis. The observation of a large number of fibroblasts would seem to support this explanation.

The unusual cell seen in the dermis (Fig. 7) resembles the polymorphonuclear heterophil described by Sandborn (52). Its presence may indicate that the polymorphs found in blood vessels at the LM level may have migrated towards the epidermis.

Even at this early stage the keratinocytes have begun to show signs of trauma (Fig. 8). The association of tonofilaments with desmosomes is reduced, as is the amount of keratohyalin present in the stratum granulosum. This suggests a disruption of the normal function of keratin production. If cellular proliferation occurs during CS (28) the cessation of keratin production may result.

The significance of the clumps of protruding cells seen in one part of the epidermis at 6 h after challenge (Fig. 9) is not readily discernable. The cells may not be associated with the CS reaction at all but a remnant of some prior epidermal activity; their identity remains obscure although they are most likely keratinocytes.

Twelve Hours Post-Challenge

Dermal mononuclear cell infiltrate is intensive, especially near hair follicles (Fig. 10). The majority of the cells resemble lymphocytes although some polymorphs are seen. To accomodate this increase in magnitude of the dermis, the epidermis is forced to bulge over heavily infiltrated areas. Some mononuclear cells are observed in the basal layers of the epidermis during LM examination and TEM observations confirm this finding (Fig. 13). The morphology of this cell - sparse clear cytoplasm, and a large dark nucleus with abundant chromatin - suggests that it is a lymphocyte and it is believed that T lymphocytes are responsible for the CS reaction (16).

Prominent vesicles are apparent in the basal epidermis (Fig. 12). These are most likely an indication of the edema that generally accompanies CS. Their diffuse contents may denote a watery substance typical of

edematous swellings. Large vesicles were not observed in the dermis at any time post-challenge.

Tonofilaments and keratinohyalin are reduced in the keratinocytes. In addition to this manifestation of CS, the stratum basale cells appear somewhat foamy. The stratum corneum can be seen to extend in pockets down into the stratum granulosum.

The basal lamina appears to be disrupted in some areas. This may be due to a general breakdown of epidermal - dermal integrity or possibly the migration of cells into or out of the epidermis.

Although no cells were observed at the junction of the dermis and epidermis, Silberberg-Sinakin (61,62) maintains that the Langerhans cell is migratory, especially in response to a CS reaction.

The large fibroblast number first noted at 6 h is still apparent at 12 h after challenge (Fig. 11). In addition, more collagen is present, indicating a function for fibroblasts in the development of induration.

Twenty-four Hours Post-Challenge

The dermal infiltration is pronounced around hair follicles (Fig. 15). The finding that the dermal invasion first appears near the hair follicle, and continues to be intensive in this area

supports the hypothesis that hair follicles are involved in the CS response. Rather than penetrating the stratum corneum, the sensitizing chemical may enter the epidermis via hair follicles or possibly sebaceous gland ducts and bind to epidermal protein carriers.

At the LM level some lymphocyte-like cells were observed in the deep dermal blood vessels. This provides evidence in support of Groth (20), who states that the infiltrating mononuclear cells are recently proliferated from cells circulating in the blood.

A continuing abundance of fibroblasts and collagen in the dermis is noted during TEM examination, and mononuclear cells can be observed situated in the superficial dermis. These may be migrating into the epidermis.

Mononuclear cells are noted in the epidermis in material embedded for LM and TEM. In addition a heterogeneous layer is present between the stratum granulosum and stratum corneum (Figs. 18,19); it contains a homogeneous substance and cellular debris. It probably represents a layer of effete keratinocytes, mononuclear cells and polymorphs sloughed off as evidenced by the eosinophil and numerous nuclei present in the matrix. It is possible that LCs are contained in this layer.

Forty-eight Hours Post-Challenge

Dermal and epidermal infiltrate, as observed by light microscopic examinations have increased to almost obliterate the epidermis in many areas (Fig. 20). Viewed by TEM this infiltrate is made up of mononuclear cells and fibroblasts and also a dark homogeneous substance which appears to occupy areas between cells and collagen. The chemical nature of this substance is unknown. It is possible that it may be the fluid which causes edema in the dermis.

The trauma to epidermal cells visible during transmission electron microscopic examination is marked (Fig. 22a). The cytoplasm is foamy and tonofilaments are lacking. The layer of debris over the stratum granulosum may be sloughing off as parts of the stratum corneum are incorporated. It is at this time that the reaction has reached a climax.

Seventy-two Hours Post-Challenge

At this stage the dermal infiltrate appears to have decreased noticeably (Fig. 25). The remaining mononuclear cells are associated with hair follicles and areas of an intensive epidermal infiltrate, possibly representing remnant cells that will migrate to the epidermis.

The epidermis appears to be at a reparative stage. It is thickened and the stratum corneum seems to extend under other parts of the epidermis, actually lifting it off (Fig. 27). This may be the ultimate significance of the downfoldings of the stratum corneum first observed at 12 h and seen at subsequent stages. The injured epidermis may be disposed of in this way.

Control - Vehicle Only

It can be concluded that the reaction observed is due to the DNFB. The dermis and epidermis exhibited no signs of trauma in the absence of DNFB either by light or electron microscopic examination (Fig. 28, 29a, b)

Control - Challenge Only

These results are more difficult to interpret. They may indicate a slight irritant reaction. However, considering the characteristics of the CS reaction, the small infiltrate observed after challenge only, cannot be viewed as a significant factor in the observed CS response. The dose of DNFB used to elicit the reaction has been documented as causing no irritant reaction (46); however, the polymorphs observed may be due in part to a slight irritant reaction not detected previously.

Considering these results it is impossible to elaborate on the function of the LC in the CS reaction. LCs were not seen in the dermis or at the junction of dermis and epidermis. In the early stages of the reaction intact LCs were observed in the basal layer of the epidermis. At later stages Langerhans cells were not noted; however, some dendrites were observed in the epidermis which belonged either to LCs or indeterminate cells. This may indicate their presence in the epidermis. Silberberg-Sinakin (61) states that mononuclear cells come into close apposition to LCs during the CS reaction. There they pick up Ag and are stimulated to fight off the invading chemical.

In order to acquire further information on the role of the LCs in the CS reaction epidermal sheets were processed for ATPase and Ia staining. At 12 h after challenge with DNFB the ATPase positive cell distribution indicates no abnormalities; the appearance of the cells has not changed from normal LCs either. Yet, at 24 h, a localization of ATPase positive cells around hair follicles exists. This is additional evidence of hair follicle or sebaceous gland duct involvement in the CS response. At 48 h the higher intensity of ATPase positive cells around hair follicles is most intensive. The interfollicular ATPase positive cells have become

increasingly round, but remain dendritic. Some of the ATPase positive cells near the hair follicle are dendritic but not all. Others are small and have round cell bodies (see Fig. 23).

To determine whether these cells are LCs which had lost their dendrites or whether they were of a different cell type, an epidermal sheet obtained 48 h after challenge was stained for Ia Ag, using anti-Ia and FITC-conjugated anti-IgG. The interfollicular distribution of Ia positive cells resembled that of normal skin (Fig. 24a). In the region of hair follicles only a slight increase in fluorescent dendritic cells was noted. It can be concluded that not all of the ATPase positive cells clumped around hair follicles are LCs. The Ia marker will stain macrophages and B cells (49) as well as LCs. Thus, the clumped ATPase positive cells could not be either of these. It is possible that the clumped ATPase positive cells are invading mononuclear cells. It is not known if ATPase stains T cells; the Ia marker is found on a very small number of T cells (49) but it is probably a subset not involved in DTH. Another possibility includes the polymorphs although their numbers are low.

An electron microscopic examination was made of the ATPase stained epidermal sheets obtained from C3H mice 48

h after challenge with DNFB. Some large cells were seen with a heavy lead precipitate associated with their cell membranes (Fig. 32). These cells possessed all of the characteristics of LCs except granules which may have been revealed by serial sectioning. It is most likely that these are LCs. In addition, some small cells with large nuclei were positively stained (Fig. 33). These cells bear a strong resemblance to lymphocytes, supporting the suggestion that it is mononuclear cells which clump around the hair follicles in epidermal sheets. Eosinophils are also seen in the epidermal sheets (Figs. 34a,b). However, they do not exhibit a positive ATPase reaction. This is additional evidence that the ATPase positive cells are mononuclear cells and not polymorphs.

It is important to note that the present study has shown that cells other than LCs cells show a positive ATPase reaction in skin challenged with DNFB. Macrophages and B cells have been eliminated as possibilities due to the fact that a correlative Ia stain (for which macrophages and B cells are positive) failed to demonstrate a corresponding distribution of Ia positive cells. The presence of ATPase negative eosinophils proves that they are not the cell in question either. It is probably invading mononuclear cells which

Figure 32 TEM. Forty-eight hours post-challenge (C3H mouse), EDTA-separated epidermis stained for ATPase: Lead precipitate (arrows) visible at membrane of a cell resembling a Langerhans cell. 3900x

Figure 33 TEM. Forty-eight hours post-challenge (C3H mouse), EDTA-separated epidermis stained for ATPase: Lead precipitate (arrows) visible at membrane of a cell resembling a mononuclear cell. 15,800x

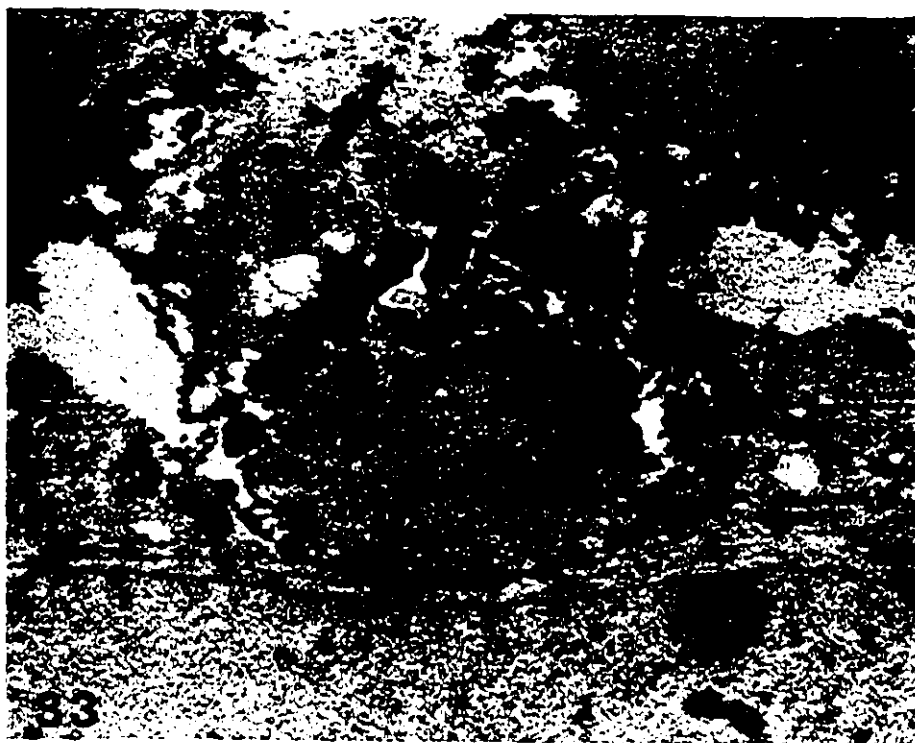
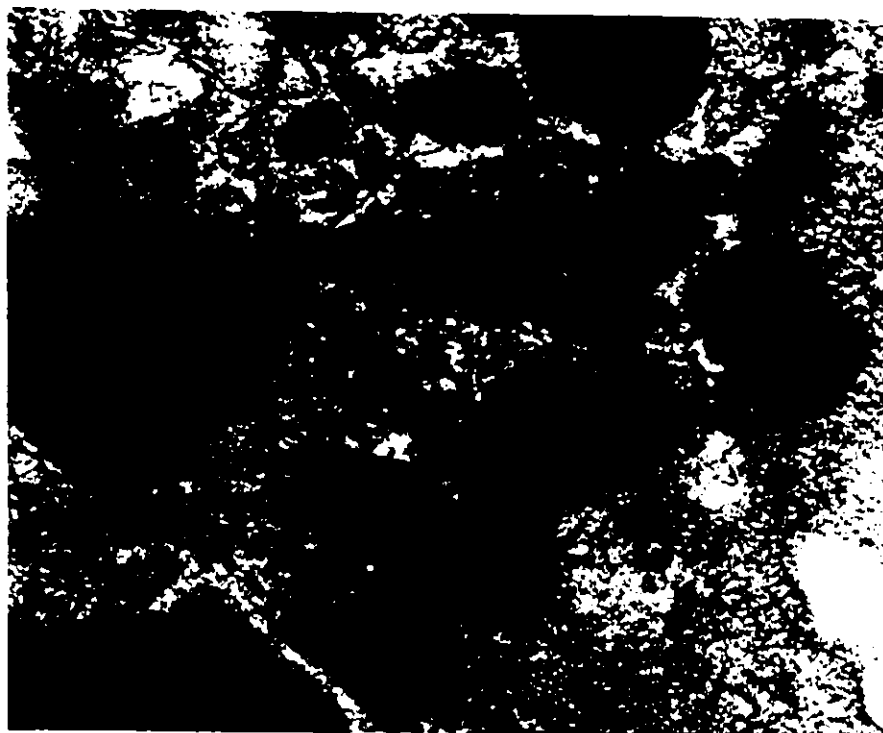
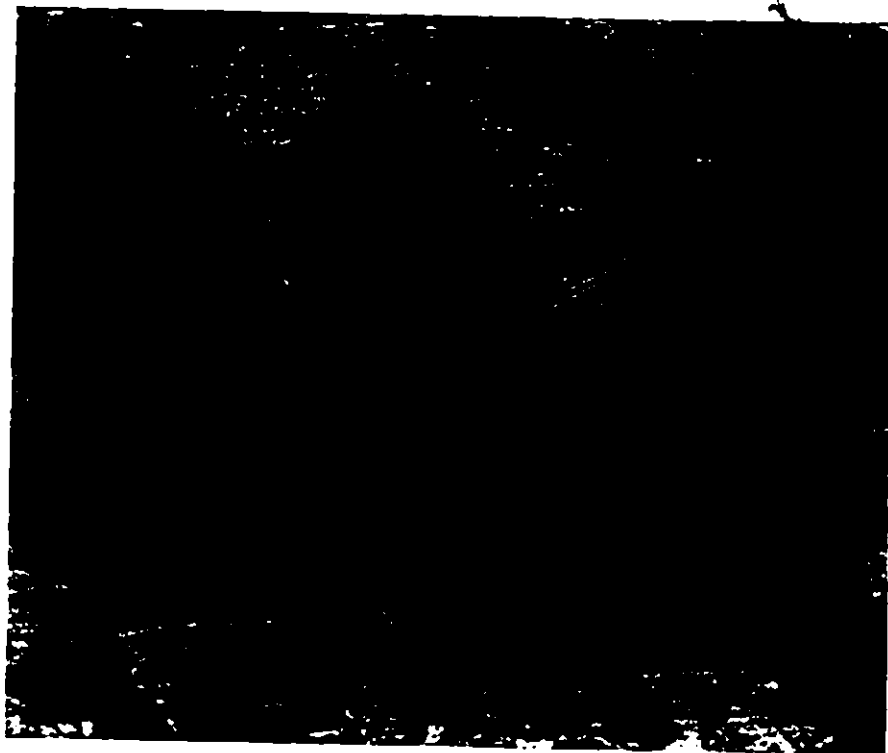


Figure 34 TEM. Forty-eight hours post-challenge (C3H mouse), EDTA-separated epidermis stained for ATPase: (a) Eosinophil (Eo) exhibits no leag precipitate at plasma membrane (arrow). 6200x (b) enlargement of eosinophil granules (gr). 57,800x

7

2



show a positive ATPase reaction.

As a control for the Ia staining an epidermal sheet was incubated in the secondary antiserum only (anti-IgG). No fluorescent dendritic cells were noted. There are a few small round cells dispersed throughout the interfollicular epidermis. Their non-specific positive stain could account for only a very small number of the Ia-positive cells seen in the 48 h sheet. In addition they do not appear to possess dendrites. The identity of these cells remains obscure. As stated they are evenly distributed in the interfollicular epidermis but they are very sparse.

It must be stated that there is a lack of evidence to support an active migratory role for LCs in the CS reaction, although this cannot be interpreted as contradicting this role. More data must be obtained on the nature of LC distributional changes in ATPase and Ia stained epidermal sheets that may occur during the course of CS. Also a thorough examination of the epidermis by transmission electron microscopy may reveal LCs in the process of migrating in or out of the epidermis or apposed to mononuclear cells.

The clearly dendritic nature of both the ATPase positive and Ia positive cells indicates that they do not undergo severe trauma as suggested by Hunziker and

Winkelmann (31). More research is required before an understanding of the functional role of LCs in the CS reaction in the mouse can be acquired.

The mouse has proved to be an ideal experimental animal in which CS can be examined. Unfortunately preliminary studies have shown that the hairless mouse, the skin of which most closely resembles that of human, is not suitable for studies involving contact skin sensitivity reactions. In haired strains the reaction does occur equally on body wall and ear skin contrary to Eisen's statement (16). The reaction to various sensitizers or under different experimental conditions can be studied. Light microscopy provides a more reliable method of determining the presence and degree of a CS reaction than measurement of ear, tail and footpad swelling.

APPENDIX I

Tissue Processing for Light Microscopy (23)

Embedding

Bouin's Fixative:

picric acid (supersaturated aqueous solution, filtered)	75 ml
formalin	25 ml
glacial acetic acid	5 ml

Schedule:

1. fix overnight in Bouin's	
2. 50% ethanol at R.T.	45 min
3. 70% ethanol " "	45 "
4. 80% ethanol " "	45 "
5. 95% ethanol " "	45 "
6. 100% ethanol at R.T.	45 "
7. 100% ethanol " "	45 "
8. 100% ethanol/benzene (1:1) at R.T.	30 "
9. benzene at R.T.	45 "
10. benzene at R.T.	45 "
11. benzene/paraffin (1:1) at 58°C	30 "
12. paraffin at 58°C	45 "
13. Paraffin, " "	45 "
14. Paraffin " "	45 "
15. embedded in fresh paraffin (melting point of paraffin 52.5°C)	

Staining

Hematoxylin (Harris') Stock Solution:

1. dissolve 1g hematoxylin in 10 ml 100% ethanol
 2. dissolve 20 g ammonium aluminum in 200 ml H₂O
 3. mix and bring to a boil quickly
 4. add 0.5g mercuric oxide
- to use: dilute 1:1 with distilled H₂O and filter.

Eosin:

1. dissolve 1g eosin in 200 ml 90% ethanol
2. adjust to pH 5.4 -5.6 by adding 8 ml 0.1 N HCl
3. filter

Staining Schedule:

xylene	5 min	0.25% HCl	30 s
xylene	5 min	tap water	5 min
xylene	5 "	50% ethanol	2 "
100%/xylene (1:1)	5 "	70% ethanol	2 "
100% ethanol	2 "	eosin	5 s
100% ethanol	2 "	95% ethanol	30 s
95% ethanol	2 "	95% ethanol	30 s
80% ethanol	2 "	100% ethanol	1 min
70% ethanol	2 "	100% ethanol	1 "
70% ethanol (55 C)	5 "	100%/xylene	2 "
50% ethanol	2 "	xylene	5 "
distilled water	2 "	xylene	5 "
hematoxylin	5 "	xylene	5 "

Toluidine Blue O

1. 1% aqueous sodium borate solution
2. 1% aqueous toluidine blue O
3. mix in 1:1 ratio
4. filter

Procedure:

1. transfer 1 u sections to a clean glass slide
2. dry at 70°C on warming plate (30 s)
3. cover sections with stain and place on warming plate for 5 s
4. drain off excess stain
5. rinse in two changes of distilled water
6. rinse in acetone
7. rinse in distilled water
8. dry on warming plate
9. mount with Permount if desired

APPENDIX II

Tissue processing for Electron Microscopy

Daltons Fixative (14)

Buffer

1. add 10.0 g potassium dichromate to 200 ml glass distilled water
2. adjust to pH 7.4 with 2.5 N KOH
3. add 250 ml 3.4% NaCl
4. add 250 ml glass distilled water

Fixative

1. 25.0 ml dichromate buffer
2. 26.0 ml glass distilled H₂O
3. 0.5 g osmium tetroxide
4. adjust to pH 7.4

Epon Embedding Medium

- all ingredients added by weight
1. tare disposable plastic cup
 2. add araldite until scale reads 34.5 g
 3. add epon 812 until scale reads 82.5 g
 4. add dodecyl succinic anhydride (DDSA) until scale reads 172.5 g.
 5. mix until homogeneous but not frothy
 6. add 3.9 ml 2,4,6-tri(dimethylamino-methyl)phenol (DMP) with a disposable syringe

Dehydration and Embedding Schedule:

1. fix tissue in Dalton's fixative - 2 h
2. 50% ethanol at 4°C - 15 min, with agitation
3. 70% ethanol " " - " " " "
4. 70% ethanol " " - overnight, without agitation
5. 90% ethanol " " - 15 min, with agitation
6. 100% ethanol at R.T. - 30 min, with agitation
7. 100% ethanol " " - " " " "
8. 100% ethanol " " - " " " "
9. propylene oxide at R.T. - 20 min, with agitation
10. propylene oxide " " - " " " "
11. 1:1 propylene oxide/epon at R.T. - 30 min, with agitation
12. 1:1 propylene oxide/epon at " - " " " agitation
13. 1:2 propylene oxide/epon at " - 60 " " agitation
14. epon medium at R.T. - 90 min, with agitation
15. embed in capsules using fresh epon medium
16. polymerize in 35-49°C oven overnight
17. polymerize in 60-70°C oven for 2 days

Uranyl Acetate Stain (UA):

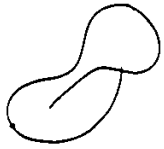
- saturated solution in 70% ethanol

Lead Citrate Stain:

1. mix 1.33 g lead nitrate, 1.76 g sodium citrate and 30 ml glass distilled water
2. shake vigorously for 1 min and allow to stand for 30 min with intermittent shaking
3. add 80 ml NaOH and dilute to 50 ml with glass distilled water
4. mix thoroughly and centrifuge if turbid

UA/Lead Citrate Staining Schedule (29)

1. place 2-3 drops UA in spot plate
2. place grid in liquid, cover to avoid light, leave for 15 min.
3. rinse grid by jetting 20 times in glass distilled water
4. let grid dry on filter paper with section side up
5. place 2-3 NaOH pellets in centre of wax filled petri dish
6. place 2-3 drops Lead citrate on wax
7. place grid in liquid, cover and leave 5 min



8. rinse grid in glass distilled water (100 ml)
with 2 drops of 10% NaOH by jetting 20 times
9. rinse grid in 2 changes of glass distilled water
by jetting 20 times in each
10. let grid dry on filter paper with section side
up

APPENDIX III

EDTA Separation and Langerhans Cell Staining

EDTA Separation (4):

1. immerse skin supported by tape backing in fresh 20 mM EDTA (50 ml phosphate buffered saline (PBS) 1% phenol and 0.372 g Na₂-EDTA.2H₂O, pH 7.3)
2. incubate for 2.5 h at 37°C
3. separate dermis from epidermis
4. rinse epidermis in PBS (R.T.) 2-5 min

Adenosine Triphosphatase Staining (4):

1. fix separated, rinsed epidermis in fresh cacodylate buffered 4% formaldehyde, pH 7.3, 20 min, 4°C
2. wash in saline solution, 10 min, at 4°C, 3 changes
3. incubate in ATP substrate solution (42 ml Tris (hydroxymethyl) aminomethane - maleic acid buffer with 8.55% sucrose, 10 mg ATP-Na₂ (Vanadium-free), 5 ml 5% Mg SO₄, 3 ml 2% Pb(NO₃)₂, added while agitating, pH 7.3), 15 min at 37°C
4. wash in saline solution, 5 min at 4°C, 2 changes
5. incubate in 1% solution of 22.3% ammonium sulphide, 20 min at 4°C

6. wash in saline solution, 5 min at 40°C, 2 changes
7. remove from tape backing, mount dermal side up, in glycerine/PBS (9:1), coverslip, and seal with paraffin

Indirect Immune Region Associated Antigen Staining:

1. fix separated, rinsed epidermis in precooled acetone, 20 min at -20°C
2. wash in PBS (pH 7.3), 15 min at R.T., 3 changes
3. incubate with mouse (strain A.TH) anti-mouse (strain A.TL) I^ak antiserum (Cedarlane Laboratories, 10:1 dilution with PBS), overnight at 37°C
4. wash in PBS (pH 7.3), 15 min at 37°C, 3 changes
5. incubate with FITC conjugated goat anti-mouse IgG (Cedarlane Laboratories, 10:1 dilution with PBS), 1 h, at 37°C
6. wash in PBS (pH 7.3, 15 min at R.T.
7. counterstain in 0.1% Evan's blue, 5 min
8. wash in PBS, 3 changes
9. remove from tape backing, mount, dermal side up, in paraphenylenediamine mounting buffer, coverslip and seal with paraffin

APPENDIX IV

Film and Print Processing

- All steps performed at 22°C

Film	Development	Rinse	Fixation	Wash
Plus x	VHD developer (Brandywine) (diluted 6:1 with tap water) 7 min	tap water	rapid fix (Kodak) (diluted 1:11 with tap water) 3 min	tap water 20 min
FGRP	D-19 (Kodak) undiluted 5 min	tap water	as above 3 min	as above 20 min
HP5	Microphen (Ilford) undiluted 7.5 min	tap water	as above 3 min	as above 20 min

Printing:

1. development: Dektol (Kodak) - 1 min at 25°C
2. rinse: tap water
3. stop bath: 2% glacial acetic acid - 30 s at 25°C
4. rinse: tap water
5. fixation: rapid fix (Kodak) - 5 min at 25°C
6. wash: tap water - 45 min to 1 h at 25°C
7. one cycle on Pakosol print drier at 95°C

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